The Role of Vitamin D in the CNS and Immune Pathology of Multiple Sclerosis

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in the undergraduate colleges of The Ohio State University.

by

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April 2021

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I. Introduction

Multiple Sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) that affects over 2.3 million individuals worldwide (1). In MS, the connection between nerves in the brain and the brain and the body, is disrupted due to the demyelination of nerve axons caused by inflammation. The CNS is essential for intaking and integrating sensory information, as well as creating a motor response for the rest of the body. Myelin is important for the efficient conduction of nerve signals along axons. In MS patients, CNS inflammation damages myelin, resulting in impaired signal conduction and the loss of sensory and motor function. The cause of the disease is unknown and there is no cure. MS is a very costly disease to both the patients and society. It is estimated that lifetime medical cost is over $4 million per patient, with a total estimated cost of $28 billion per year in the US, second only to heart disease (2). While the clinical course of MS has been studied extensively, the preclinical phase of MS is largely unstudied (3). Low Vitamin D (VitD) during childhood has been identified as a risk factor for MS (4). With new mouse models and technology, it is possible to study the role of VitD during early life and determine the impact that low VitD has on the risk of developing MS (5-13). Given that VitD is an inexpensive dietary supplement and readily available via sunlight, it provides an avenue to develop preventative measures against the disease.

Multiple Sclerosis

MS is most often diagnosed in young adults between 20-40 years of age with females 3 times more likely to develop MS than males (14-15). The clinical course of the disease is diverse with

symptoms ranging from numbness to paralysis, and approximately 1 in 4 MS patients becoming confined to a chair or bed during their lifetime. The initial symptoms are caused by immune-mediated demyelination which impairs axonal transmission. The myelin sheath is an insulating biological material found on the axon of neurons, and it increases the speed of action potentials along the axon. Without a myelin sheath, nerve impulses are incomplete or less efficient throughout the body resulting in a loss of neural communication in the brain and to the peripheral nervous system (PNS). When myelin is damaged or destroyed, scarring occurs in the CNS, further interrupting impulses potentiating along axons. A lack of myelination between neurons often causes symptoms such as muscular weakness, tingling, fatigue, spasticity, difficulty walking, vision problems, dizziness, and others. Symptoms are varied by individual cases, and symptoms and the severity of symptoms vary throughout the course of the disease. Fortunately, remyelination occurs after the inflammation subsides and symptoms often improve resulting in a relapsing-remitting disease course during the initial phase of the disease. Over the course of the disease, axons become damaged resulting in permanent functional deficits. At this point in disease, patients have continued progression of neurological symptoms and are defined as having secondary progressive MS. Loss of axons has been correlated with brain atrophy and has become a major focus in understanding the pathology of MS. Current therapies are aimed at minimizing the inflammatory response, but there are no current therapies to aid in axonal repair (16).

Inflammation in MS

MS is the result of a malfunction of the body's immune system which results in CNS

inflammation and ultimately the destruction of the myelin sheath. MS is thought to be an autoimmune disease. In other words, the body's immune system attacks the myelin sheath. The immune system is divided into two parts: innate and adaptive. The adaptive immune system has two important immune cells: T cells and B cells (**Fig. 1**) (17). T cells and B cells have unique receptors on their cell surface that recognize specific proteins. During infections, these receptors can bind to proteins (also known as antigens) of microorganisms and specifically target the microorganism for destruction through various mechanisms. T cells can directly kill infected host cells, activate other immune cells, produce cytokines and inflammatory mediators, and regulate the body's immune response. B cells primarily produce antibodies against specific antigen and act as antigen presenting cells to T cells. T cells and B cells are critically important in the clearance of pathogens because they have specificity for a pathogen, and they have memory. This means a small population of T cells and B cells that have specificity for a pathogen will remain after the infection and protect from re-infection.

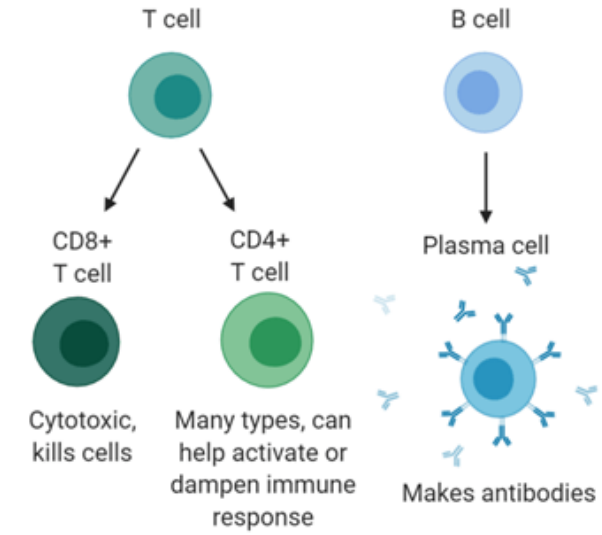

Figure 1: Types of T and B cells

Figure 1: Types of T and B cells (17).

In MS, T cells and B cells enter the CNS via the blood brain barrier. There are 2 major types of T cells: CD4 and CD8. T cells only recognize antigens bound to MHC molecules. Proteins are broken into peptide fragments, bound to MHC I or MHC II molecules [as known as Human Leukocyte Antigens (HLA)], and presented on the cell surface of cells to T cell receptors (**Fig. 2**). CD8 T cells recognize peptides bound to MHC I and are largely responsible for lysing infected cells. CD4 T cells recognize peptides bound to MHC II and are also called T helper cells because they 'help' other immune cells do their job. CD4 T cells can be divided into several subsets, including Th1, Th2, Th17 and Tregs. Th1 cells and Th17 cells that have receptors for myelin proteins are thought to be the pathogenic cells in MS (18). Th1 cells produce inflammatory cytokines such as IFNγ, TNFα, and GM-CSF. Th17 cells produce IL-17 and GM-CSF. These cytokines can activate other immune cells, particularly B cells and innate immune cells. These inflammatory cytokines, as well as other inflammatory mediators, are thought to damage myelin. In a healthy immune system, there are regulatory T cells (Tregs) which monitor inflammation and the immune response. Tregs do not function properly in patients with MS, causing the immune system to continue its attack on the myelin sheath. CD4 T cells also aid B cells in antibody production and recruiting other immune cells to the CNS. While antibody production is a major role of B cells in infection, it is thought that B cells are important antigen presenting cells in MS. Depletion of B cells in MS patients is beneficial, while depletion of antibodies has minimal benefit (18).

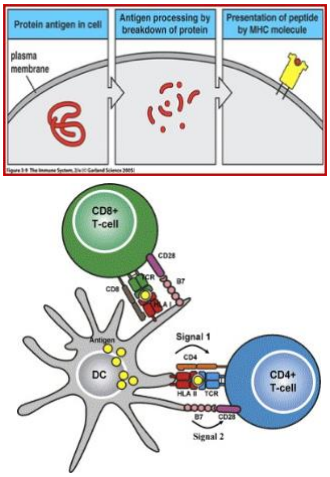

Figure 2: Dendritic cells with antigen presenting MHCI/II molecules binding peptides to different T cell receptors.

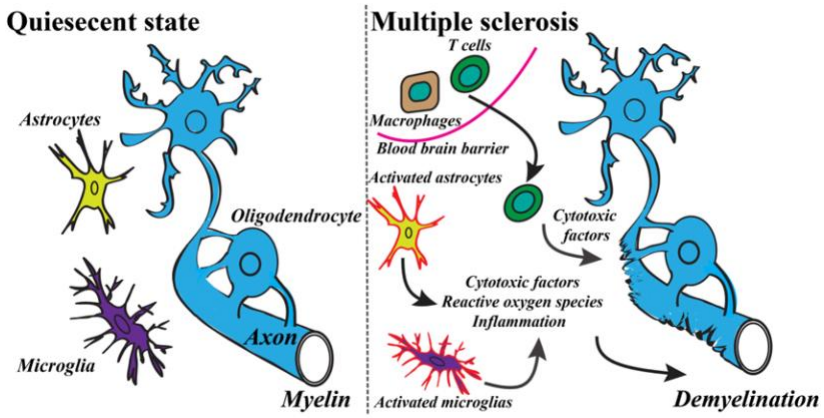

Figure 3: Typical CNS and the Demyelination of Axon in MS (20).

The innate immune system also contributes to MS pathology. As T cells disrupt the blood brain barrier, innate immune cells also cross into the CNS and are present in MS lesions. In addition to B cells, macrophages and dendritic cells (innate immune cells), are important antigen presenting cells for the activation of CD4 T cells. Innate immune cells act quickly in response to infection by recognizing molecular structures that do not exist in humans, such as specific, unique sugars

or lipids that are present on the microorganisms. Macrophages and dendritic cells differentiate from monocytes in the bloodstream. When monocytes identify areas of infection or damage, they enter the tissue and become macrophages or dendritic cells. The cells are phagocytic and engulf and destroy infected tissue and cells. Macrophages and dendritic cells produce an array of inflammatory cytokines and inflammatory mediators. In MS, macrophages and dendritic cells contribute to tissue damage by recruiting additional inflammatory cells to the myelin sheath and secreting inflammatory cytokines that damage myelin (**Fig. 3**). It is known that VitD has anti-inflammatory properties (21). Given that low VitD in childhood increases the risk of developing MS as an adult, it was thought that low VitD may be causing a dysregulated immune system that predisposes one to the development of MS (5-13).

Vitamin D

Vitamin D (VitD) is an inexpensive, fat-soluble nutritional supplement that could be readily increased in the diet of children to minimize the risk of developing MS. VitD is found in many foods, however the amount of VitD found in food is low, even when provided as a supplement (100 IU/cup milk). Sunlight is the best source of VitD, which is produced when UVB radiation converts 7-hydro-cholesterol in the skin to VitD (**Fig. 4**) (22). A 20-minute sun exposure can produce 10-fold the amount of VitD as a cup of supplemented milk. With the increase of screen time in young children in the US, time outside in the sun is limiting resulting in lower production of VitD. This factor, in addition to low VitD found in foods, makes studying the impact of VitD in the CNS and immune system even more pressing as a potential risk factor for MS and other

neurodegenerative diseases like Parkinson's, that could be easily prevented with supplementation (21).

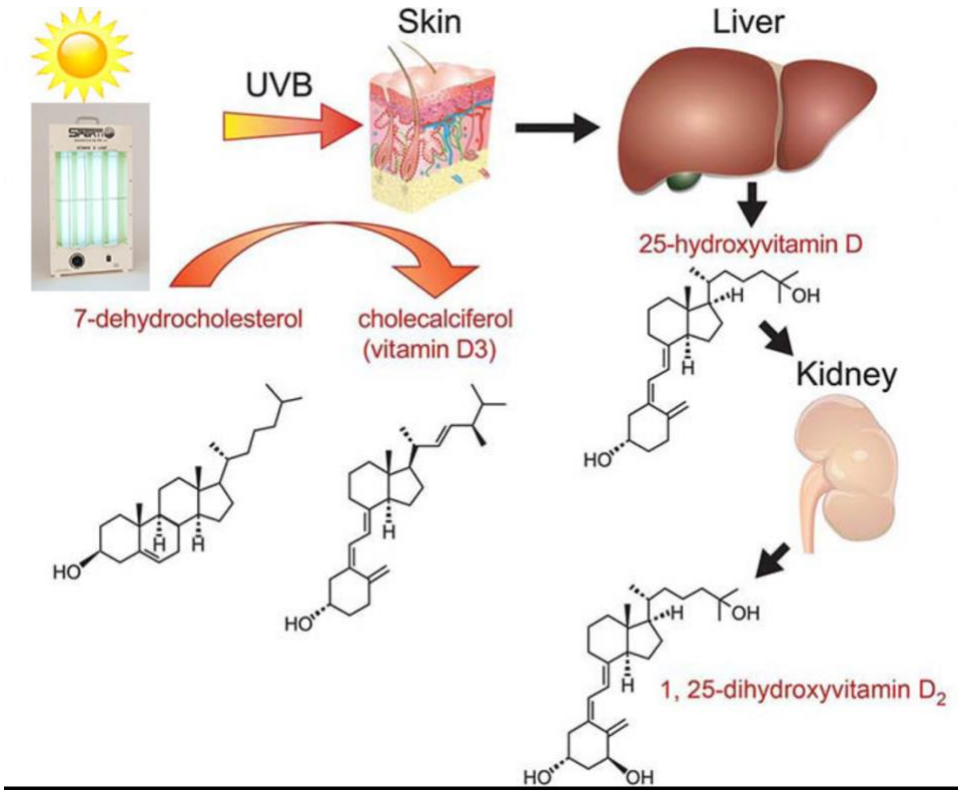

Figure 4: Vitamin D production pathway and absorption from sunlight (23).

VitD is essential for gut absorption of calcium and is necessary for bone growth and remodeling by osteoclast and osteoblasts (19). Without VitD, bones can become brittle, and the risk of bone deterioration is high. Other responsibilities of VitD include reducing inflammation and modulating cell growth and glucose metabolism. VitD is an essential vitamin in developing babies and children, as well as in adults (5-13). Vitamin D Receptors (VDR) are widely expressed on many tissues and cell types, including immune cells and CNS cells. VDR-deficient

mice display abnormal CNS development and altered immune function (24-25), confirming that VitD is a vital player in both of these systems.

Immune and CNS cells have (VDR) on the cell surface and have the ability to synthesize active VitD (24-25). Resident CNS cells, neurons, oligodendrocytes, astrocytes and microglia express VDR, yet there is little data about the role of VDR signaling in these cell types (26-32). It has been recently demonstrated that both CNS and innate immune cells express the enzyme (1α-hydroxylase) necessary to produce the active form of VitD (calcitriol) (30, 33). Thus, both the immune system and CNS have the capacity to produce and respond to VitD. Deficiency of VitD is also correlated with other autoimmune diseases like rheumatoid arthritis, diabetes mellitus, and inflammatory bowel disease. In a Parkinson disease model, VitD was shown to protect neurons via reduced microglial activation (34). VitD has been shown to protect neurons by upregulating GDNF (35). By targeting a lack of VitD during development, and observing the effects on MS, easy preventative measures could be taken against MS in areas with low sunlight. VitD supplements during early childhood could prevent the development of MS and other neurodegenerative diseases later in life if neuroprotectant. Looking at the distribution of patients with MS, those closer to the equator have a lower incidence of the disease and areas farther north and south of the equator have a higher incidence of MS (15), supporting the hypothesis that sufficient VitD levels are necessary for normal CNS and immune development. Studying the role of VitD in immune-mediated diseases, such as MS, is important for developing a potentially simple and effective supplementation of VitD during childhood in a society where time outside is quickly decreasing.

Central Nervous System

The cells of the CNS consist of neurons and four types of glial cells: astrocytes, oligodendrocytes (first oligodendrocyte precursor cells (OPCs)), microglia, and ependymal cells. The role of astrocytes is to regulate the CNS and control flow of molecules across the blood brain barrier; they serve as a first line of defense against invasion in the CNS. Ependymal cells are a structural glial cell that line the ventricles and spinal canal and produce cerebrospinal fluid. Neurons serve to pass information through the nervous system and communicate with other cells. The axon is the area of the neuron that carries the action potential to the dendrites of the next neuron. Myelin surrounds the axons and acts as an insulator for the conduction of neural impulse. Oligodendrocytes myelinate axons during development and maintain the myelin sheath in the CNS. OPCs are responsible for remyelinating axons that has been damaged. During MS, OPCs migrate to lesions, differentiate into oligodendrocytes, and remyelinate axons. Over time, scaring occurs at lesion sites making it difficult for OPCs to migrate to areas of demyelination, resulting in permanent loss of myelin and often axonal severing. Microglia are resident macrophages of the CNS and protect neurons from infection. Since microglia are phagocytic and can act as antigen presenting cells, they can re-activate CD4 T cells in the CNS and likely promote lesion formation. Additionally, activated microglia produce cytokines and inflammatory mediators, such as reactive oxygen species, that can directly damage myelin. Because the myelin sheath has been damaged by immune cells, neurons are not able to communicate in MS. Oligodendrocytes are also destroyed in MS which prevents the repair of myelin and communication is further cut off between neurons. Apoptosis of oligodendrocytes triggers a response from microglia and more myelin is destroyed. The result of neurodegeneration in the CNS leads to the symptoms observed in MS due to a lack of communication in the CNS.

Modeling MS

Experimental autoimmune encephalomyelitis (EAE) is an in vivo model of MS, characterized by immune inflammation and demyelination of the CNS (36). While EAE can be induced in many types of animals, mice are the most commonly used. EAE can be induced in multiple strains of mice by immunizing the mice with a myelin protein or peptide combined with Complete Freund's Adjuvant (CFA). The CFA contains *Mycobacterium tuberculosis* (Mtb) which will be readily recognized by innate immune cells at the site of immunization. This will provoke dendritic cells and macrophages to phagocytose the Mtb and the myelin protein/peptide. The dendritic cells will migrate to the lymph nodes with the myelin protein/peptide where T cells with T cell receptors with specificity for the protein/peptide become activated. Pertussis toxin injections are given to aid in the breakdown of the blood brain barrier which allows the immune response to attack the CNS. These myelin-specific T cells will then traffic to the CNS and initiate inflammation and demyelination. These inflammatory lesions in EAE look very similar to MS lesions. EAE displays high levels of demyelination in white matter. The clinical course of the disease varies between different mouse strains. SJL/J mice develop relapsing/remitting EAE and the disease course most similar to MS. In active EAE, 75%-80% of immunized mice can be expected to develop disease (36). EAE can also be induced by transferring myelin-specific CD4 T cells from mice that have been immunized with myelin proteins/peptides into naïve mice. In SJL/J mice, transfer of activated myelin-specific T cells will result in the recipient mice developing relapsing-remitting EAE similar to the immunization of mice SJL/J mice.

To study the effects of VitD deficiency on the risk of developing MS, we utilized an in vivo system, known as tamoxifen-inducible Cre/l*oxP*, system that allowed us to delete the VDR from

specific cell types at specific time points in SJL/J mice. Cre recombinase is an enzyme that cuts at *loxP* sites. *LoxP* sites can be added to the end of genes that are targeted for deletion. In the case of VDR, *loxP* sites were added to the VDR gene, now known as the floxed gene (VDR^{fl}) (37). Cre can be transgenically added to mice using cell-specific promoters so that cre will only be expressed in a specific cell type. To allow for the induction of cre at specific time points, mice with a tamoxifen-inducible cre system were used. In the inducible cre system, the cre gene is fused with a mutant estrogen receptor gene that prevents cre from accessing the nucleus of cells. When the mice are fed tamoxifen (an estrogen receptor antagonist), cre can enter the nucleus and cut out the gene that has *loxP* sites (**Fig. 5**) (38). In the case of VDR, the VDR^{fl} mice can be crossed with a specific tamoxifen-inducible cre mouse. These mice will be normal because the cre cannot access the nucleus. However, when tamoxifen is given to the mice, cre will translocate to the nucleus and cut the *loxP* sites that surround the VDR gene. Thus, these mice can now express VDR when tamoxifen is not present. For this study, we used CD4-creER to target CD4 T cells, CD19-creER to target B cells, CX3CR1-creER to target microglia, PLP1-creER to target oligodendrocytes, and CSPG4-creER to target OPCs. Since our intent was to generate VitD insufficiency, not total deletion, the creER mice were crossed with $VDR^{f/+}$ mice, which meant that one copy of the VDR gene had floxed sites but the other VDR gene was normal. The creER X $VDR^{f/+}$ mice should have partial deletion of VDR, mimicking VitD insufficiency. Since our interest was understanding how VitD insufficiency in early life was important to the risk of developing MS, we administered tamoxifen in the chow at 3-5 weeks of age, equivalent to 6-12 years of age in humans. This will generate mice that have normal VDR expression at birth, but upon tamoxifen-feeding, VDR expression will be partially lost during early life.

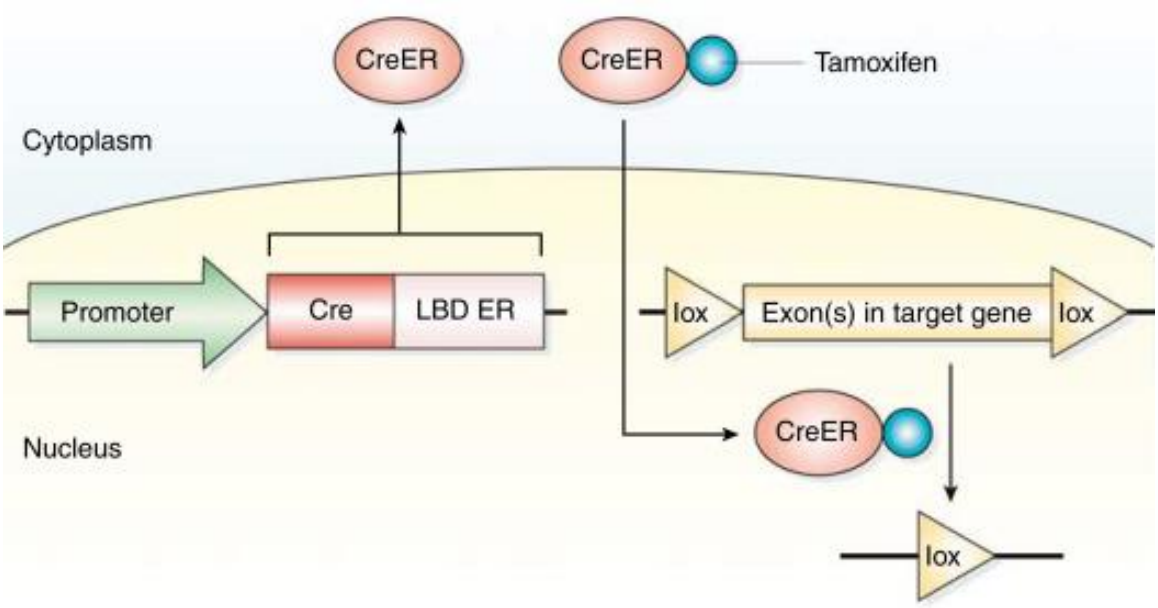

Figure 5: Tamoxifen Inducible *Cre*-lox system (39)

Hypothesis and Objective

MS is a disease without a cure and no known cause. This leads us to study the role of VitD in the immune system and CNS with relationship to MS during development. The main hypothesis is that reduced VitD levels during early life makes the CNS more vulnerable to inflammation, enhancing the susceptibility to autoimmune demyelinating disease. This study is rationalized by the potential results of correlating early life VitD deficiency to MS susceptibility that could be the basis of creating a public health policy to account for the easily modifiable risk factor for MS. To determine if VitD insufficiency in the CNS and immune system in juvenile mice enhances susceptibility to MS, a mouse model was utilized. Using the tamoxifen-induced cre-*lox* system, the VDR was partially deleted to mimic VitD insufficiency in T cells (CD4-creER), B

cells (CD19-creER), microglia (CX3CR1-creER), oligodendrocytes (PLP-creER), and OPCs (CSPG4-creER) during development. At adulthood, these mice underwent suboptimal EAE induction to determine if low VitD signaling in early life, in a specific CNS or immune cell population, enhanced EAE susceptibility. It was predicted that mice with partial VDR deletion during development would have an earlier onset of EAE and a more severe course of disease than those mice with a normal CNS and immune phenotype.

This study is a critical step in understanding the impact of low VitD levels during childhood as a risk factor for MS. The contribution of VitD signaling in the immune system and CNS is necessary for understanding the role VitD plays in MS and how the data could be applied to other autoimmune and neurodegenerative diseases.

II. Methods and Materials

Generating VDR^{fl} X creER mice

Swiss VDR^{fl} mice were acquired from Geert Carmeliet (40) and backcrossed with SJL/J mice. Similarly, CD4-creER, CD19-creER, CX3CR1-creER, PLP-cre-ER, and CSPG4-creER mice, all purchased from Jackson Laboratories, were backcrossed >8 generations to SJL/J mice. Then VDR$^{f/+}$ mice were crossed with each of the creER mouse lines. The offspring included VDR$^{f/+}$creER^{+}, VDR$^{+/+}$creER^{+}, VDR$^{f/+}$creER^{-}, and VDR$^{+/+}$creER^{-} mice. The pups were weaned

at 3 weeks of age and fed tamoxifen chow until 5 weeks of age. This allowed the CNS to develop properly, but also allowed for VitD depletion during juvenile development. The *cre-lox* system allows for tissue specific and inducible knockout. *Cre+* mice contain the cre recombinase transgene that has a tissue specific promoter. "Floxed" mice have two *loxP* sites that flank a gene of interest, in this case VDR. When fed tamoxifen, the cre recombinase transgene was activated and the floxed gene of interest was excised (37).

EAE Induction and Monitoring

Test ($VDR^{f/+}creER^{+}$) and control ($VDR^{+/+}creER^{+}$, $VDR^{f/+}creER^{-}$, and $VDR^{+/+}creER^{-}$) mice at 10 weeks old were immunized with a peptide emulsion to induce sub-optimal EAE. Sub-optimal EAE was induced so that we could observe if there was an increase in the incidence of EAE or a more severe disease course in the test mice (41). Emulsion was made with myelin peptide known to induce EAE in SJL mice (Proteolipid Protein 139-151) (100ug/mouse), Complete Freund Adjuvant (CFA) with H37Ra as the medium for the emulsion, and additional heat-killed mycobacterium tuberculosis (1 mg/mL) to activate an immune response. The emulsion was injected on Day 0 in four sites of the mouse, above the hind flanks and front arms, near the axial and inguinal lymph nodes. An intraperitoneal pertussis toxin injection (200 ng/mouse) was given on Day 2 to help break down the blood brain barrier and allow the myelin-specific T cells to enter the CNS (42). At Day 6, mice were observed daily for signs of neurological deficits and scored for disease severity until Day 60. Scoring guidelines can be found in **Table 1**. The daily scores for mice were graphed and groups are compared.

Table 1: Clinical EAE Scores (42)	
Clinical Score	Description
0	Healthy, normal mouse. No changes in motor function compared to non-immunized mice. When picked up by the tail, the tail has tension and motion control. Hind legs are spread apart and there is not affect to the gait or head tilt when moving.
1	Tip of tail or whole tail is limp. When picked up by the tail the tail drapes over finger and does not curl or have motion control. Hind limps are still strong.
2	Limp tail and moderate hind limb weakness. When picked up by the tail the legs are held closer together. When the mouse is walking, the gait is wobbly. One foot may have the toes drag, but there is no paralysis.
3	Limp tail and severe hind limbs. Both hind limbs are able to move but are very weak. Mouse in unable to maintain grasp of cage bars with hindlimbs when flipped upside down.
4	Limp tail and complete hind limb paralysis. When placed on the bottom of cage, mouse is only able to move its front limbs during movement. Hind limbs have no motion, legs are splayed, and toes drag.
5	Limp tail, hind limb paralysis, and partial front limb paralysis. Mouse in minimally moving around the cage but is alert and feeding. Mice often euthanized if a score of 5 persists or the mouse is severely dehydrated/malnourished.
6	Mouse is found dead due to paralysis or mouse is euthanized due to moribund state.

Evaluation of Gene Knockdown

To confirm the knockout of VDR using the *cre-lox* system, mice were fed tamoxifen for 2 weeks beginning at 3 weeks of age to mimic the set-up of the EAE experiments. The brain or spleen of the mice were removed. For brain, the tissue was dissociated using a GentleMACs instrument and the Miltenyi Biotec protocol for mouse brain tissue dissociation (43). Neurons were

separated from tissue using the Miltenyi Biotec protocol for isolation of mouse neurons with non-neuronal cell beads and a LS column (44). Microglia were separated from tissue with the Miltenyi Biotec protocol for mouse microglia isolation via CD11b microbeads and a MS column (45). OPCs were separated using the Miltenyi Biotec protocol for mouse OPC isolation with CD140a microbeads and a MS column (46). To detect the knock-out of VitD from $VDR^{fl/+}$-creER mice, PCR was run to show that the VDR gene was removed from these mice. This was done with Real Time PRC, which uses fluorescence to track the differences between populations and is highly sensitive to differences between samples. RNA was extracted from each cell type and cDNA was synthesized to use for Real Time PCR with a VDR primer and Hprt housekeeping gene, used to detect differences between wildtype and $VDR^{fl/+}$-creER mice. If the VDR gene was removed from the $VDR^{fl/+}$-creER mice, the measured fluorescence of these mice should be less than the fluorescence of wildtype mice with the VDR gene. Insufficient data was collected at the time of writing this paper for evaluating the knock down of VDR via the *cre-lox* system. This part of the study is ongoing.

Statistics

Clinical EAE scores were analyzed with Prism software. For each experiment, the mean clinical scores and standard error were graphed for 60 days. Test mice were defined as expressing VDR-flox and Cre. The control mice were littermates that had all the other possible genotypes for VDR-fl and/or Cre which would not result in a loss of VDR expression. To evaluate the frequency of disease, all mice that had a clinical EAE score of 3 or above were considered ‘sick’.

Mice were also defined as ‘sick’ if they scored a 1 or 2 more than 5 times. A healthy mouse could be scored as a 1 or 2 if the mouse was lethargic and not performing well for the scoring test. In analysis, all mice categorized as ‘sick’ had a clinical EAE score of 3 or above or score a 1 or 2 more than 5 times. This method of separation attempted to account for this factor, as well as sick mice that were never as severe as a 3. The Mann-Whitney Test was used to calculate a p-value and determine significance between test and control groups. The Mann-Whitney Test is nonparametric, and the null hypothesis makes the assumption that the distributions of both populations are equal. This test was used because the difference between clinical EAE scores is subjective. Differences between populations were significant if the p-value was less than .05.

III. Results

CD4 T cells

In MS, it is known that myelin specific CD4 T cells mediate the disease, and VitD has been shown to reduce CD4 T cells activation and function. Therefore, we analyzed whether partial deletion of the VDR gene in young mice would enhance their risk of developing CNS autoimmunity. To do this, we crossed VDR^{fl} mice with CD4-creER mice, and fed the pups chow with tamoxifen for 2 weeks (3-5 weeks of age). The breeding was done such that the test mice were $VDR^{fl/+}$CD4-$creER^{+}$, so that the mice had a partial deletion of VDR, but not complete loss of VDR. When the mice were 10 weeks old, EAE was induced by immunization with PLP/CFA. Overall, CD4 mice with partial VDR knockout did not have more severe disease, did not develop

EAE earlier, and did not have a longer disease course than control mice as a general trend (**Fig. 6A**). The p-value for all CD4 mice was 0.7264 indicating that the difference between test and control mice was not significant. There were 15 test mice and 51 control mice. Out of these test mice, 11 mice (73.3%) were defined as sick, and 29 (56.9%) of the control mice were defined as sick. This data suggests that VDR signaling in CD4 T cells in early life is not critical in preventing CNS autoimmunity.

While combined data sets for the CD4 T cells is negative, there were individual experiments that supported the hypothesis that VDR signaling in CD4 T cells in early life may be important in preventing CNS autoimmunity. Experiment 643 A/B showed that there was a significant difference between were $VDR^{fl/+}$CD4-$creER^{+}$ and control mice with a $p<0.0001$ (**Fig. 6B**). Thus, it may be necessary to investigate VDR signaling in CD4 T cells more thoroughly to definitively determine if VitD insufficiency in early life alters CD4 T cells and CNS autoimmunity.

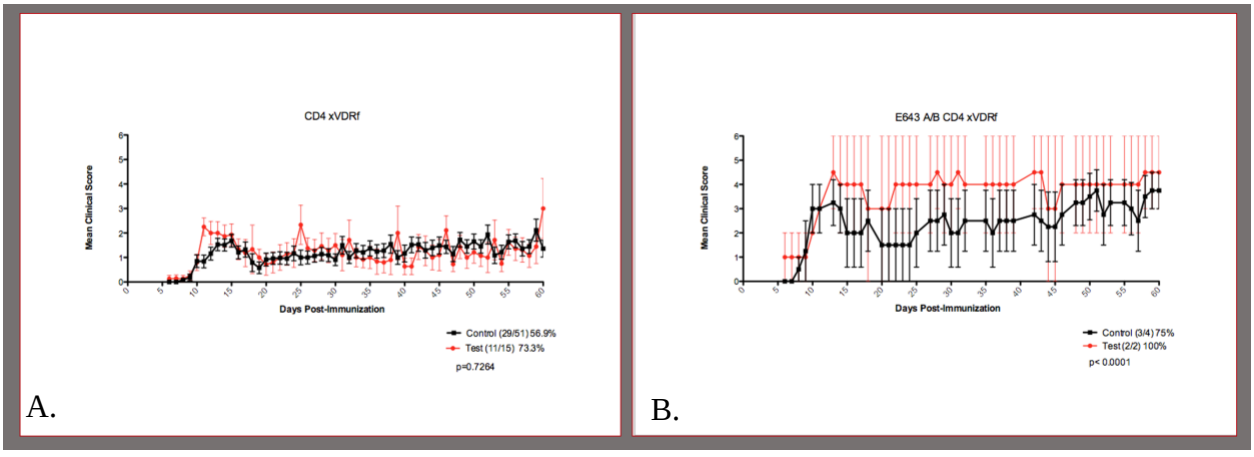

Figure 6: **Analysis of EAE in mice with VDR partial deletion in CD4 T cells. A**. Graph of all CD4 experiments (23) showing no significant difference in $VDR^{fl/+}$CD4-cre^{+} and wildtype mice p=0.7264. **B**. Graph of experiment 643A/B showing $VDR^{fl/+}$CD4-cre^{+} mice as sicker than wildtype mice with a $p<0.0001$.

CD19

In MS, it is known that B cells play a role in the disease because therapies that cause B cell depletion are beneficial in MS patients (47). In order to determine if VitD insufficiency in B cells may contribute to MS risk, we analyzed whether partial deletion of the VDR gene in B cells in young mice would enhance their risk of developing CNS autoimmunity. To do this, we crossed VDR^{fl} mice with CD19-creER mice, and fed the pups chow with tamoxifen for 2 weeks (3-5 weeks of age). The breeding was done such that the test mice were $VDR^{fl/+}$CD19-$creER^{+}$, so that the mice had a partial deletion of VDR, but not complete loss of VDR. When the mice were 10 weeks old, EAE was induced by immunization with PLP/CFA. CD19 mice with partial VDR knockout displayed more severe disease and had a longer disease course than control mice as a general trend (**Fig. 7A**). The p-value for all CD19 mice was <0.0001 indicating that the difference between test and control mice was significant. There were 7 test mice and 13 control mice. Out of these test mice, 7 (100%) were defined as sick, and 10 (76.9%) of the control mice were defined as sick. This data supports the hypothesis that VDR signaling in B cells in early life is important in preventing CNS autoimmunity.

As a combined group, data on CD19 B cells supports the importance of VDR signaling in B cells in relation to autoimmunity. Individual experiments, like 609E (**Fig. 7A**), indicate that $VDR^{fl/+}$CD19-$creER^{+}$ mice sicker than control mice with $p<0.0001$. Overall, the trends of $VDR^{fl/+}$CD19-$creER^{+}$ mice compared to control mice indicate that CD19 B cells are important for VDR signaling and development of CNS autoimmunity.

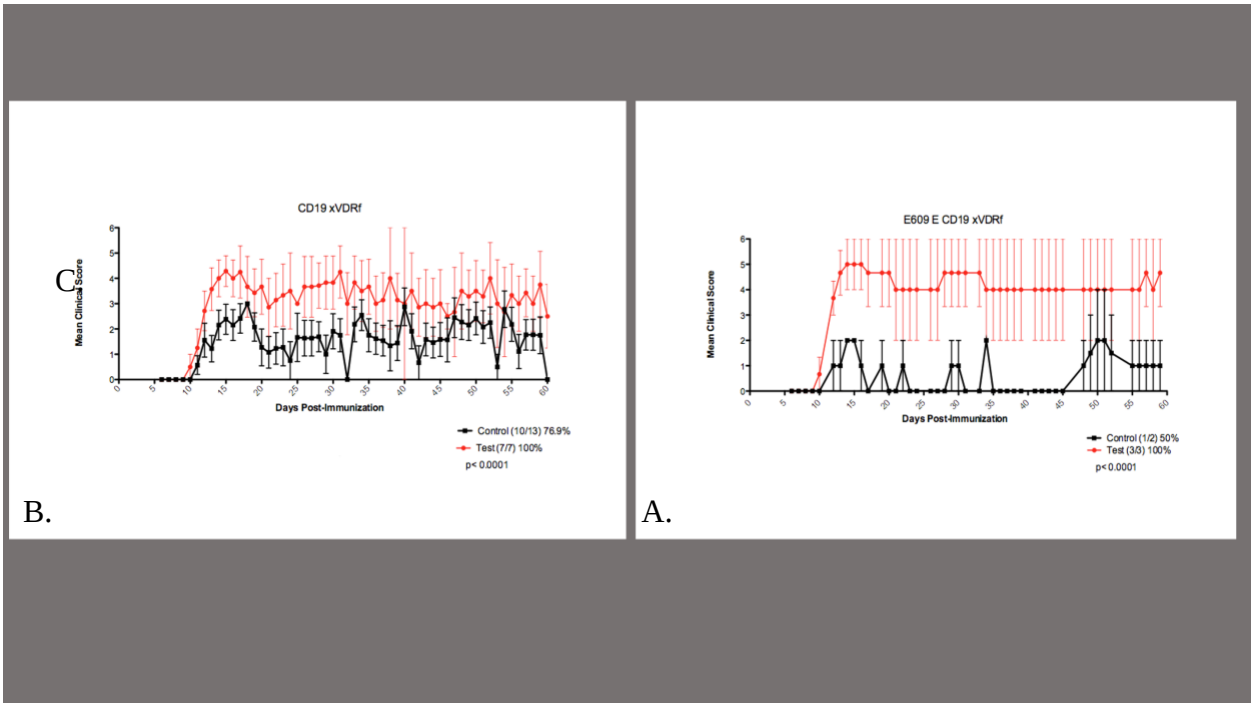

Figure 7: Analysis of EAE in mice with VDR partial deletion in B cells. A. Graph of all CD19 experiments (6 experiments) showing $VDR^{fl/+}CD19\text{-}cre^{+}$ mice sicker than wildtype mice with $p<0.0001$. **B.** Graph of E609 E showing $VDR^{fl/+}CD19\text{-}cre^{+}$ mice sicker than wildtype mice with $p<0.0001$.

Oligodendrocytes

It is known that oligodendrocytes do not function properly when maintaining the myelin sheath in MS allowing for reduced signal conduction. VDR is also a receptor on oligodendrocytes. Therefore, we analyzed whether partial deletion of the VDR gene in oligodendrocytes in young mice would enhance their risk of CNS malfunction and developing inflammation. To do this, we crossed VDR^{fl} mice with PLP1-creER mice, and fed the pups chow with tamoxifen for 2 weeks (3-5 weeks of age). The breeding was done such that the test mice were $VDR^{fl/+}PLP1\text{-}creER^{+}$, so that the mice had a partial deletion of VDR, but not complete loss of VDR. When the mice were 10 weeks old, EAE was induced by immunization with PLP/CFA. PLP1 mice with partial VDR

knockout displayed more severe disease and had a longer disease course than control mice as a general trend, however $VDR^{fl/+}$PLP1-$creER^{+}$ mice had a delayed onset of disease compared to control mice. (**Fig. 8A**). The p-value for all PLP1 mice was 0.0014 indicating that the difference between test and control mice was significant. There were 6 test mice and 19 control mice. Out of these test mice, 5 (83.3%) were defined as sick, and 16 (84.2%) of the control mice were defined as sick. This data supports the hypothesis that VDR signaling is important in preventing CNS malfunction in oligodendrocytes. The difference is evident in the severity of the disease course, but not in the incidence of disease.

As a combined group, data about oligodendrocytes supports the importance of VDR signaling in CNS function. However, experiment 561B (**Fig. 8B**) and experiment 640C (**Fig. 8C**) do not agree with this combined result. In experiment 561B, control mice were sicker than $VDR^{fl/+}$PLP1-$creER^{+}$ mice with $p<0.0001$. Experiment 640C did not show a difference between $VDR^{fl/+}$PLP1-$creER^{+}$ mice and control mice. Individual experiments, like 647D (**Fig. 8D**), indicate that $VDR^{fl/+}$CD19-$creER^{+}$ mice were sicker than control mice with $p<0.0001$. Studies like these indicate the importance of continued research about the role of VDR signaling in oligodendrocytes.

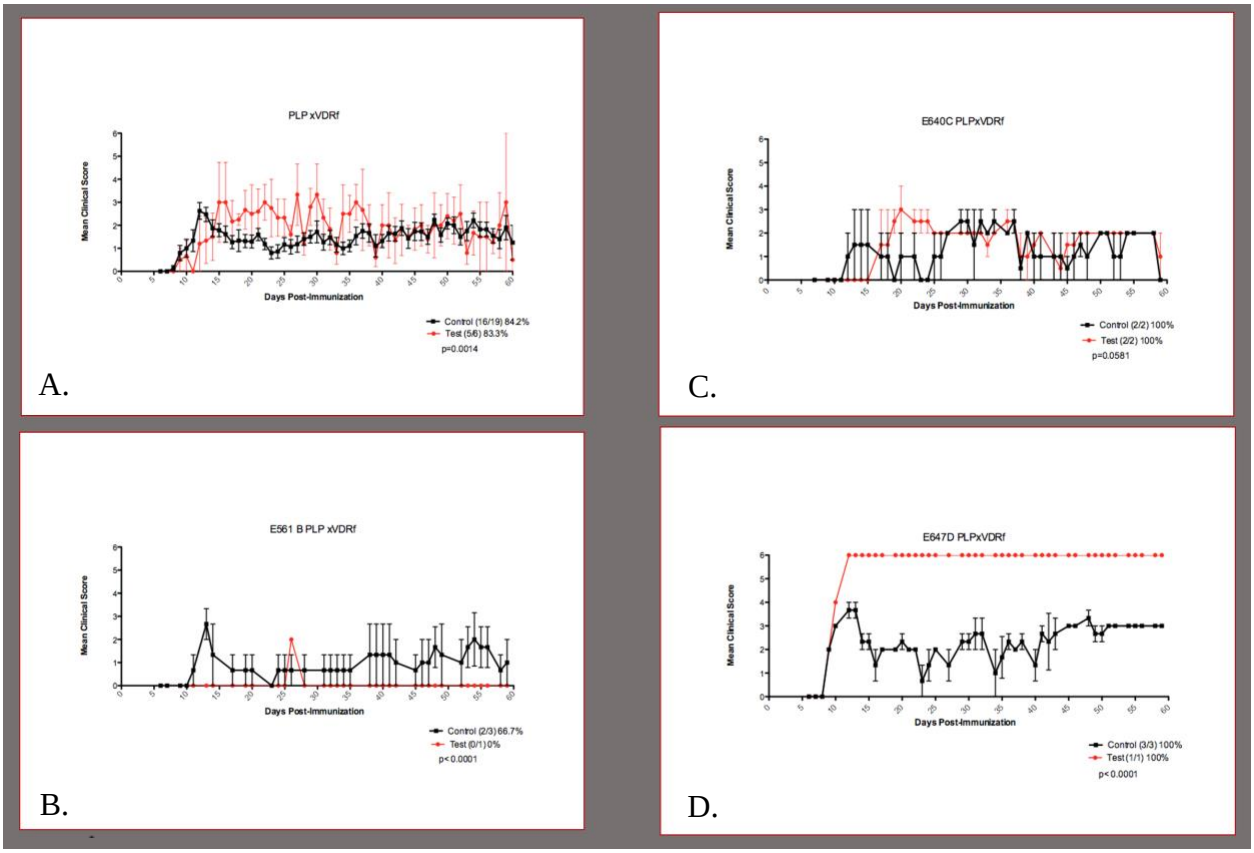

Figure 8: Analysis of EAE in mice with VDR partial deletion in oligodendrocytes. A. Graph of all PLP1 experiments (9 experiments) showing $VDR^{fl/+}PLP1\text{-}cre^{+}$ mice as sicker than control mice with p=0.0014. **B.** Graph of experiment 561B showing wildtype mice as sicker than $VDR^{fl/+}PLP1\text{-}cre^{+}$ mice with p<0.0001. **C.** Graph of experiment 640C showing no clear difference between $VDR^{fl/+}PLP1\text{-}cre^{+}$ mice and control mice with p=0.0581. **D.** Graph of experiment 647D showing $VDR^{fl/+}PLP1\text{-}cre^{+}$ mice as sicker than control mice with p<0.0001.

OPCs

OPCs are required for remyelination of lesions in MS. Early in MS, and in relapsing-remitting EAE, remyelination occurs. However, the ability to effectively remyelinate axons declines during the course of disease. VDR is also a receptor on OPCs. Therefore, we analyzed whether partial deletion of the VDR gene in OPCs in young mice would enhance their risk of CNS malfunction and developing inflammation. To do this, we crossed VDR^{fl} mice with CSPG4-creER mice, and fed the pups chow with tamoxifen for 2 weeks (3-5 weeks of age). The breeding

was done such that the test mice were $VDR^{fl/+}$CSPG4-$creER^{+}$, so that the mice had a partial deletion of VDR, but not complete loss of VDR. When the mice were 10 weeks old, EAE was induced by immunization with PLP/CFA. CSPG4 mice with partial VDR knockout displayed more severe disease and had a longer disease course than control mice as a general trend (**Fig. 9A**). The p-value for all CSPG4 mice was 0.0006 indicating that the difference between test and control mice was significant. There were 18 test mice and 45 control mice. Out of these test mice, 11 (61.1%) mice were defined as sick, and 29 (64.4%) of the control mice were defined as sick. This data supports the hypothesis that VDR signaling is important in preventing CNS malfunction in OPCs.

As a combined group, data about OPCs supports the importance of VDR signaling in OPCs in normal CNS function. Individual experiments, like 572A/B/C (**Fig. 9B**), indicate that $VDR^{fl/+}$CD19-$creER^{+}$ mice were sicker than control mice with $p<0.0001$. Experiment 575 B/C (**Fig. 9C**), also support this observation with $p=0.0007$. Overall, the trends of $VDR^{fl/+}$CSPG4-cre^{+} mice compared to wildtype mice indicate that VDR signaling in OPCs is important for CNS function.

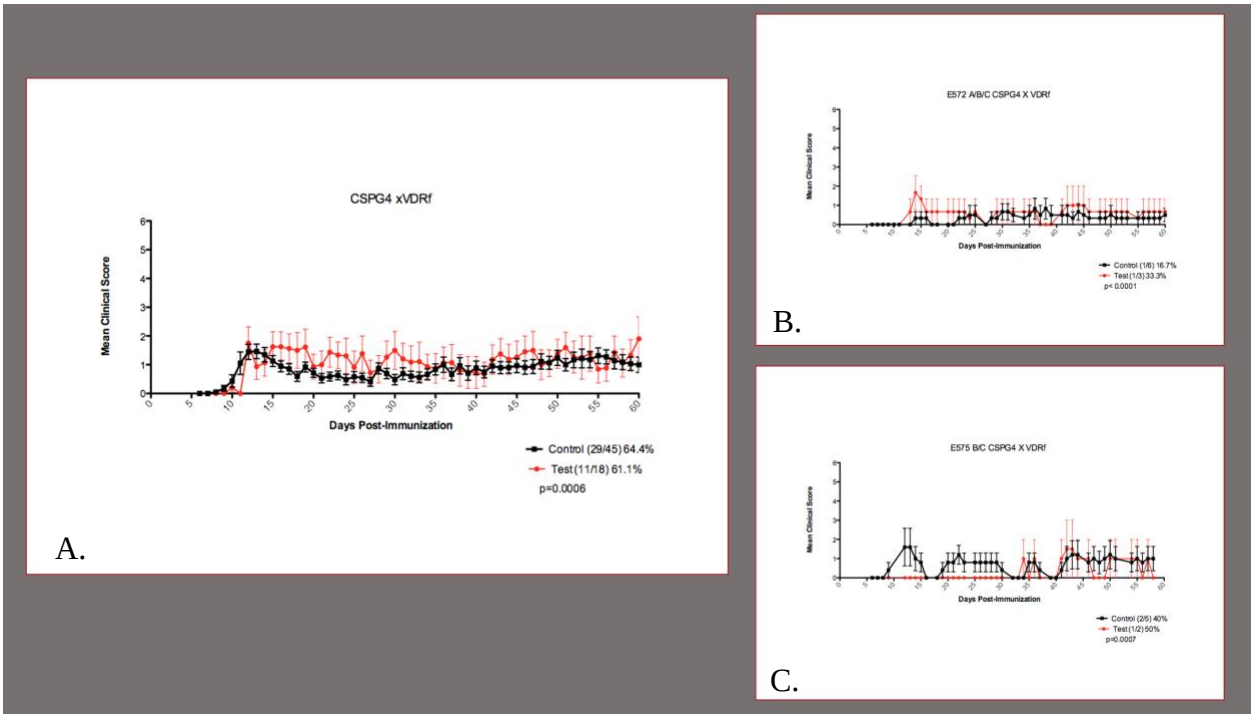

Figure 9: A**nalysis of EAE in mice with VDR partial deletion in OPCs. A.** Graph of all CSPG5-cre experiments (22 experiments) indicating that $VDR^{fl/+}$CSPG4-cre^{+} mice were sicker than control mice with p=0.0006. **B.** Graph of experiment 572A/B, showing $VDR^{fl/+}$CSPG4-cre^{+} mice as sicker than control mice with p<0.0001. **C.** Graph of experiment 575B/C, showing a significant difference between $VDR^{fl/+}$CSPG4-cre^{+} mice and control mice with p<0.0001.

CX3CR1

Microglia are known to be a major contributor to the inflammatory process in the lesions in MS and EAE. Therefore, we analyzed whether partial deletion of the VDR gene in microglia in young mice would enhance their risk of developing CNS malfunction and inflammation. To do this, we crossed VDR^{fl} mice with CX3CR1-creER mice, and fed the pups chow with tamoxifen for 2 weeks (3-5 weeks of age). The breeding was done such that the test mice were $VDR^{fl/+}$CX3CR1-$creER^{+}$, so that the mice had a partial deletion of VDR, but not complete loss of VDR. When the mice were 10 weeks old, EAE was induced by immunization with PLP/CFA. CX3CR1 mice with partial VDR knockout displayed more severe disease than control mice as a

general trend (**Fig. 10A**). The p-value for all CX3CR1 mice was <0.0001 indicating that the difference between test and control mice was significant. There were 17 test mice and 39 control mice. Out of these test mice, 10 (58.8%) defined as sick, and 25 (64.1%) of the control mice were defined as sick. This data supports the hypothesis that VDR signaling is important in preventing CNS malfunction in microglia.

As a combined group, data about microglial cells supports the importance of VDR signaling in microglia in relation to a normal CNS. Individual experiments, like 621 A/B (**Fig. 10B**), indicate that $VDR^{fl/+}CX3CR1\text{-}cre^{+}$ mice were sicker than wildtype mice with p<0.0001. Overall, the trends of $VDR^{fl/+}CX3CR1\text{-}creER^{+}$ mice compared to control mice indicate that VDR signaling in microglia is important for function of the CNS.

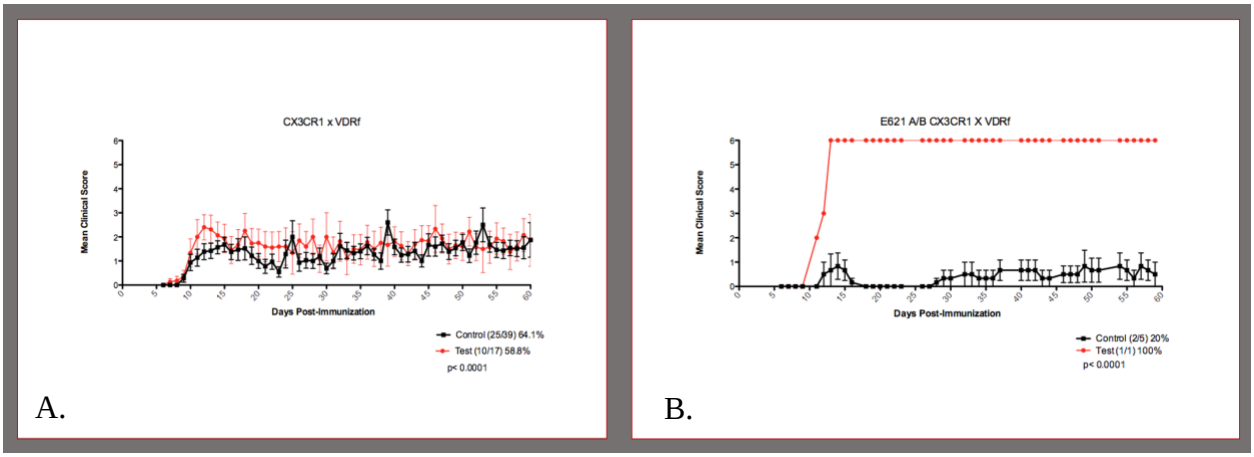

Figure 10: Analysis of EAE in mice with VDR partial deletion in microglia. A. Graph showing all CX3CR1 experiments (22 experiments) indicating that $VDR^{fl/+}CX3CR1\text{-}cre^{+}$ mice were sicker than control mice with p<0.0001. **B.** Graph of experiments E621A/B, indicating that $VDR^{fl/+}CX3CR1\text{-}cre^{+}$ mice were sicker than control mice with p<0.0001.

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IV. Discussion

Multiple sclerosis is an autoimmune inflammatory disease of the CNS. The cause of MS is unknown; however, studies have shown that a lack of VitD in children may be a risk factor for developing MS (5-13). Given that VDRs are expressed in both the immune system and CNS, this study was designed to provide insight into the relevance of VitD in MS susceptibility. In this experimental study, we looked at the role of VDR signaling in the immune system, more specifically in CD4 T cells and CD19 B cell, and in the CNS (microglia, oligodendrocytes, and OPCs) in relation to mouse susceptibility to and disease course of EAE.

The role of VDR signaling in the adaptive immune system is partially conclusive. In CD19 B cells, a lack of VDR in $VDR^{fl/+}$CD19-$creER^{+}$ mice resulted in a higher percentage of mice developing EAE after immunization compared to control mice. This difference was significant when comparing all 6 VDR/CD19-creER experiments. When experiments were analyzed individually, some experiments displayed the same conclusion drawn from all VDR/CD19-creER experiments. However, some experiments did not have a significant p value for differences between $VDR^{fl/+}$CD19-cre^{+} mice and control mice. Using all mice from the VDR/CD19-creER experiment in analysis, the conclusion can be drawn that VDR signaling in CD19 B cells is important because $VDR^{fl/+}$CD19-cre^{+} mice had more severe disease than control mice. This data suggests that B cells that developed with VitD deficiency may promote inflammation in the CNS. B cells play several roles in inflammation. In the context of MS, it is thought that B cells major role is as antigen presenting cells, since antibodies do not appear to be a major factor in MS. It is known that B cell depletion is beneficial in patients with MS (47). Our data may indicate that low VitD makes B cells more efficient antigen presenting cells to pathogenic or autoreactive T cells. More data on how VitD signaling regulates B cell function is

needed to fully understand the mechanism. Additionally, the conclusion that VitD deficiency in B cells causes atypical B cell development or function may be easily remedied with a VitD supplement during development of children in areas with less sunlight.

The role of VDR signaling in CD4 T cells, the cells mediating damage to the myelin sheath is less conclusive. When drawing conclusions from all 23 VDR/CD4-creER experiments, there is not a significant difference between $VDR^{fl/+}$CD4-cre^{+} mice and control mice. A general trend observed on **Fig. 4A** is that $VDR^{fl/+}$CD4-cre^{+} mice were more severely sick initially, but the disease course of $VDR^{fl/+}$CD4-$creER^{+}$ mice and control mice was similar, with an insignificant p value. Experiment 643 show that $VDR^{fl/+}$CD4-$creER^{+}$ mice were sicker than control mice with statistical significance. Other experiments support this trend as well, however there are statistically significant experiments that show control mice sicker than $VDR^{fl/+}$CD4-$creER^{+}$ mice. These differences in experiments and groupings of VDR/CD4-creER mice do not indicate that VDR signaling in CD4 T cells is significant. Future studies about the role of VDR signaling should be done to draw firm conclusions about the role of VitD deficiency in T cell function because T cells are an important cell involved in the autoimmunity of MS. Because T cells and B cells are team players in the adaptive immune system, altering VDR signaling in one cell type may affect the function of the other cell type. Thus, it would be important to have a deeper study into T cell function in mice with VDR insufficiency in B cells, T cells, or both cell types.

In the CNS, the reduction of VDR during development impacted microglia, oligodendrocytes, and OPCs. For each of these CNS studies, mice with VDR reduction were sicker than control mice. $VDR^{fl/+}$PLP1-$creER^{+}$ and $VDR^{fl/+}$CSPG4-$creER^{+}$ mice were sicker than control mice, however they became sick after control mice when comparing all experiments for each line. For all three CNS cell types, there are statistically significant individual experiments that also

showed VDR$^{fl/+}$-creER^{+} mice sicker than control mice. The observation that partial VDR knockout mice are sicker than control mice indicates that VitD is important when the CNS is developing. Because of this, it can be concluded that a VitD supplements could be used as a preventative measure for developing autoimmune diseases like MS.

Data indicating that VitD is an essential vitamin for B cells and CNS development is consistent with the disease distribution pattern of MS. MS frequently occurs in populations farther from the equator where there is less sunlight to produce VitD (15). Combining this observation with experimental evidence of the negative impact a depletion of VitD has on EAE offers a causal relationship between MS and VitD. Additionally, the B cell study indicates that VDR signaling is important to have healthy B cell function (47). Other studies have shown that reducing B cells in patients reduces the symptoms of MS. Confirming that VitD depletion leads to increased B cell function means therapies could be used to target B cells with VitD, or this process could be prevented with VitD supplementation.

A major limitation to this study is power within experiments due to breeding constraints. Breeding SJL/J mice is difficult because of behavioral factors, and this makes it difficult to have a large number of VDR$^{fl/+}$-creER^{+} and control mice in a single study. Because of this, data was primarily analyzed using the large group of all mouse experiments in a line which may have variation between experiments due to the longitudinal aspect of this study. Individual experiments were also analyzed with a small number of mice, sometimes only one control or VDR$^{fl/+}$-creER^{+} mice would be in this analysis. To overcome this, breeding of VDR$^{fl/+}$-creER^{+} mice needs to be increased by mating more parents, however this is expensive and takes time.

Verifying VDR knock down in $VDR^{fl/+}$-$creER^{+}$ mice has been another limitation to this study. Unless knockdown is verified, the study is only assuming that $VDR^{fl/+}$-cre^{+} mice have a partial deletion of VDR. Without verification, conclusions are not significant. The first approach to verifying the efficiency of the cre-lox system was using immunohistochemistry. $VDR^{fl/+}$-$creER^{+}$ and control mouse neural tissue was removed, fixed, and sectioned with a microtome. Tissue was stained for VDR receptors using unconjugated antibodies and viewed under a fluorescent microscope. Results between $VDR^{fl/+}$-$creER^{+}$ and control mice did not show a difference. This was attributed to the non-specific antibody used and not a lack of genetic difference between genotypes. Next, a challenge was deciding how to verify the VDR knockdown. Preliminary work had been done using Real Time PCR to detect difference in mRNA VDR levels to distinguish between $VDR^{fl/+}$-$creER^{+}$ and control mice. A challenge associated with this approach is isolating these small number of CNS cells. A tissue dissociation and cell separation protocol (43-46) were combined to isolate neurons, microglia, and OPCs. After analysis with Real Time PCR, data for VDR knockdown in $VDR^{fl/+}$-creER mice was not significant, but very few samples were available. Because of this, future experiments must be performed using more mice to confirm the deletion of the VDR gene in $VDR^{fl/+}$-creER mice. Improvements on the cell separation method above could include confirming that the separated cells are the target population, and that the population is pure. This could be done using Flow Cytometry to quantify and identify the cell types with cell-specific antibodies.

Looking forward, the role of VDR signaling should be studied in other cell types, double floxed mice, and CD4 T cells. While CD4 T cells were studied in this paper, more work to understand the role of VDR signaling in the most important immune cells mediated MS should be done to

come to a firm conclusion about the importance of VitD during development for these cells. Because previous research has shown CD4 T cells as the mediator of MS, research should be done to definitively conclude VitD's role in these cells seeing as a VitD supplement could be a simple preventative measure to cell malfunction.

In this study, we used $VDR^{fl/+}$-$creER^{+}$ mice with a partial VDR knock down. A possible question for this study is: is there a VitD threshold for MS susceptibility? Using $VDR^{fl/fl}$-$creER^{+}$ mice would mean a full knockdown of VDR. With our previous theories about the importance of VitD for healthy CNS and immune function, we would hypothesis that a $VDR^{fl/fl}$-$creER^{+}$ mouse would be sicker than control mice or $VDR^{fl/+}$-$creER^{+}$ mice because of the lack of VitD. Additionally, the time point of VitD deficiency could be varied to understand if there is a specific point where VitD is more important for development. Data has been collected, but not finished about time points and full VDR knockout mice.

Another course of action is to study the importance of VitD signaling in other CNS and immune cells. For example, monocytes in the immune and astrocytes and all neurons in the CNS could be used in a study about VDR signaling. Work has begun on these lines but is incomplete. Monocytes are an important cell to study because they are innate immune cells that differentiate into dendritic cells and macrophages, other immune cells that contribute to the destruction of the myelin sheath in MS. In addition, these cells are antigen presenting cells to CD4 T cells and can impact the activation and function of myelin-specific T cells. In the CNS, it is important to study neurons because they are the main cell of the system; if there is an important supplemental factor that could increase healthy neuronal cells, this would be a simple preventative measure to

neurodegenerative diseases. In a paper by Lee, et. al. 2020, it was determined that VitD has anti-inflammatory effects in neurons by inducing anti-inflammatory molecules like Hmox1 and Arg1. The study indicates that calcitriol (active form of VitD) could induce the production of molecules in neurons that could reduce pro-inflammatory molecules and promote anti-inflammatory molecules in microglia (48). In addition, mice that had reduced VDR in neurons had a reduced severity of EAE. Lastly, astrocytes are CNS cells that help regulate the blood-brain barrier (where immune cells enter the brain) and inflammation in neural cells (49). Astrocytes are a key cell in MS and may also be impacted by VDR signaling. There are many other important cells that play roles in the disease pathology of MS that could be studied to understand the importance of VitD deficiency and MS susceptibility.

Overall, we studied the role of VitD in the immune system and CNS with relationship to MS. The main hypothesis was that reduced VitD levels during early life makes the CNS more vulnerable to inflammation, enhancing the susceptibility to autoimmune demyelinating disease. To study this, we used a tamoxifen-induced *cre-lox* system, where the vitamin D receptor (VDR) was partially deleted to mimic vitamin D insufficiency in T cells (CD4-creER), B cells (CD19-creER), microglia (CX3CR1-creER), oligodendrocytes (PLP-creER), and OPCs (CSPG4-creER) during early life. We determined that lack of VitD caused mice to be sicker when it was removed from B cells, microglia, oligodendrocytes, and OPCs. Entire data sets about CD4 T cells are inconclusive, however individual data sets have indicated an importance of VitD in CD4 T cells. Due to these findings, we are able to conclude that a VitD supplement during development in children is a simple, preventative measure against MS because we have shown that a lack of VitD increases severity of disease.

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